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Exhibit A

Correlation of a Common Mutation in the Methylenetetrahydrofolate Reductase Gene With Plasma Homocysteine in Patients With Premature Coronary Artery Disease

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Abstract Mild hyperhomocysteinemia, a risk factor for occlusive arterial disease, can be caused by disruptions of homocysteine metabolism. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-methyltetrahydrofolate, the methyl donor for homocysteine remethylation to methionine. A common mutation in *MTHFR*, an alanine-to-valine substitution, may contribute to mild hyperhomocysteinemia in coronary artery disease (CAD). To test this hypothesis, we studied 152 patients with CAD by mutation analysis, *MTHFR* enzymatic assays, and measurements of plasma homocysteine and several vitamins. The *MTHFR* mutation was associated with reduced enzymatic activity and increased enzyme thermolability in these patients. The difference in the prevalence of the homozygous mutant genotype between the CAD patients (14%) and an unmatched group of healthy subjects (10%) was not significant. However, individuals with the homozygous mutant genotype had higher plasma homocysteine, particularly when plasma folate was below the median value. This genetic-environmental interaction is proposed to be a risk factor for CAD. (*Arterioscler Thromb Vasc Biol.* 1997;17:569-573.)

Key Words • homocysteine • methylenetetrahydrofolate reductase • folic acid • genes • mutation

Mild hyperhomocysteinemia has been identified as a risk factor for occlusive arterial disease. Several case-control studies have demonstrated that patients with vascular disease have fasting homocysteine concentrations that are ≈30% higher on average than those of control subjects.[1-4] In one prospective study, elevated homocysteine (>95th percentile) was associated with a threefold increase in the risk of acute myocardial infarction.[5] A recent report from the Framingham Heart Study has suggested that the risk of carotid artery stenosis of >25% was increased in subjects with homocysteine concentrations previously considered to be in the normal range.[6]

Hyperhomocysteinemia can result from genetic or nutrient-related disturbances of homocysteine metabolism. Homocysteine can be transsulfurated to form cysteine or remethylated to form methionine. The latter reaction uses 5-methyltetrahydrofolate as a carbon donor; 5-methyltetrahydrofolate is synthesized from 5,10-methylenetetrahydrofolate through the action of MTHFR. Kang et al[7] first reported that a thermolabile variant of *MTHFR* was present in 17% of North American patients with CAD. More recently, this variant was reported in Dutch patients with several forms of vascular disease.[8]

We have isolated the cDNA for human *MTHFR* and described 10 mutations in this gene.[9-11] Nine of these mutations are rare; they were identified in patients with severe MTHFR deficiency, an inborn error of folate metabolism with pediatric or adolescent onset of neurological and vascular symptoms.[9,10] One common mutation, an alanine-to-valine substitution, has been expressed in vitro and results in thermolabile *MTHFR*.[11] In a small group of individuals, we showed that the mutation correlated with reduced enzymatic activity and increased thermolability in lymphocyte extracts. Individuals who were homozygous for the mutation had increased levels of plasma homocysteine. In this report, we examine the prevalence of *MTHFR* genotypes and show a correlation between genotype and biochemical phenotype (enzyme activity and plasma homocysteine levels) in French Canadian patients with premature CAD. More importantly, we suggest an interaction between the homozygous mutant genotype and folate levels in the development of hyperhomocysteinemia.

Methods

Subjects

The patients (121 men and 31 women), all French Canadians, were monitored at the Cardiology Clinic of the Clinical Research Institute (Montreal, Quebec) or referred from the Cardiology Service of the Hotel-Dieu Hospital (Montreal). All patients had angiographically documented CAD (>50% stenosis of a major epicardial coronary artery) or had suffered a myocardial infarction documented by enzymatic and electrocardiographic criteria. The patients were all younger than 60 years of age at the time of sampling, which was performed in

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Selected Abbreviations and Acronyms

CAD = coronary artery disease
CBS = cystathionine-β-synthase
CH_2THF = [^{14}C]CH_2-tetrahydrofolate
FAD = flavin adenine dinucleotide
MTHFR = 5,10-methylenetetrahydrofolate reductase
PCR = polymerase chain reaction
SAM = S-adenosylmethionine

the free-living state at least 8 weeks after discharge. A group of French Canadians (86 men and 35 women), white-collar workers from a major utility company, were selected on the basis of being healthy and free of major CAD risk factors except for cigarette smoking. Both patients[12] and healthy subjects[13] have been described elsewhere.

Genetic Analysis

DNA was isolated from peripheral leukocytes with phenol chloroform after cell lysis in a sucrose buffer.[14] Screening for the 677C→T substitution (A to V) was performed by PCR of genomic DNA, followed by *Hin*fI digestion and polyacrylamide gel electrophoresis, as previously described.[11]

Measurements of Enzyme Activity and Thermolability

Peripheral mononuclear white blood cells were isolated by the method of Böyum[15] from 10 mL of blood that had been collected in tubes containing EDTA as an anticoagulant. The cell pellets were stored at −70°C for <4 weeks. On thawing, the mononuclear white blood cells were lysed by addition of 150 μL 0.05% Triton X-100 in 0.1 mol/L potassium phosphate buffer. Enzyme activity was determined in the reverse direction by a modification of the method of Rosenblatt and Erbe[16] as follows: Cell extracts were incubated for 60 minutes at 37°C in a reaction mixture containing 0.18 mol/L phosphate buffer, 3.5 mmol/L menadione, 1.4 mmol/L EDTA, 7.6 mmol/L ascorbic acid, 70 μmol/L FAD, and 300 μmol/L [^{14}C]CH_2THF in a total volume of 143 μL. For assessment of thermostability, the reaction mixture containing lymphocyte extract and all other components except [^{14}C]CH_2THF and FAD was preincubated at 46°C for 5 minutes before the addition of substrate and FAD. The reaction was terminated by the addition of 125 μL 0.6 mol/L sodium acetate, pH 4.5. After the addition of 50 μL 100 mmol/L formaldehyde and 75 μL 0.4 mol/L dimedone, the mixture was boiled for 12 minutes and subsequently cooled on ice. To each sample, 2.5 mL toluene was added, and the tubes were vigorously vortexed twice for 15 seconds. After centrifugation of the samples for 10 minutes at 1000 rpm, formation of the radiolabeled [^{14}C] formaldehyde-dimedone adduct was quantified by scintillation counting of the supernatant. Enzyme activity was expressed as nanomoles of formaldehyde formed per hour per milligram of protein. The formation of formaldehyde was shown to be linear with time and with concentration of protein in the reaction mixture in a range that included the concentrations used for these analyses (0.05 to 0.1 mg protein per assay). Protein was determined by the method of Lowry using bovine serum albumin as standard.

Determination of Plasma Homocysteine and Vitamins Involved in Homocysteine Metabolism

Homocysteine refers to total homocyst(e)ine, ie, in its reduced form, as homocystine or as the homocysteine-cysteine mixed disulfide, free or protein bound. Fasting blood samples were collected in EDTA-containing tubes and kept on ice. Plasma was separated within 2 hours of sampling by centrifugation (20 minutes, 4°C, 3000 rpm) and multiple 1-mL aliquots were stored at −70°C for further studies. Total plasma homocysteine was determined by high-pressure liquid chromatography, according to the method of Araki and Sako.[17] Plasma folic acid was determined by a microbial assay, vitamin B_{12} with a commercial radioimmunoassay kit, and pyridoxal-5-phosphate by the tyrosine decarboxylase method as previously described.[18]

TABLE 1. Biological Parameters in a Group of Healthy Subjects (n=121) and in Patients With CAD (n=152)

	Healthy Subjects	CAD Patients
Age, y	41.6±5.3	48.8±6.6*
Total cholesterol, mmol/L	5.3±0.9	6.4±1.5*
VLDL cholesterol, mmol/L	0.68±0.4	1.3±0.9*
LDL cholesterol, mmol/L	3.3±0.8	4.1±1.4*
HDL cholesterol, mmol/L	1.3±0.3	0.9±0.2*
Triglycerides, mmol/L	1.1±0.7	2.7±1.8*
Homocysteine, μmol/L	9.6±4.8	10.8±3.2*
Folate, ng/mL	4.2±2.7	4.7±3.0 (121)
Vitamin B_{12}, pg/mL	311.9±118.5	308.3±134.8 (81)
Pyridoxal phosphate, pmol/mL	46.9±37.8 (109)	30.9±25.4 (102)*
Males/females, n/n	86/35	121/31

Values are mean±SD. Values in parentheses indicate number of tested individuals for parameters that were not tested in the entire group.
*$P<.05$ between healthy subjects and CAD patients.

Statistical Analyses

Comparison of MTHFR activities between groups was performed by one-way ANOVA, and the association between MTHFR activity and thermolability was examined by use of the Spearman's rank correlation test (nonparametric ANOVA). Two-tailed probability values are given, and $P<.05$ was considered significant. Comparison of biological parameters between control subjects and patients was done by use of Student's *t* test. Genotype distributions were examined by χ^2 analysis.

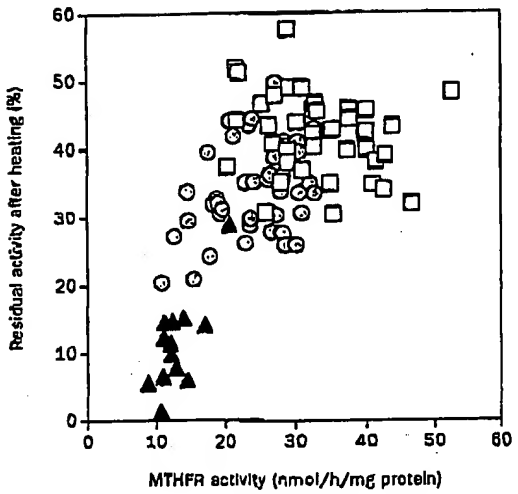
Results

Table 1 indicates the relevant characteristics of the CAD group and the group of healthy subjects. Homocysteine levels were significantly higher ($P<.05$) in the CAD group. The groups did not differ significantly with respect to plasma folate or vitamin B_{12} levels, but plasma pyridoxal phosphate was significantly lower in the CAD group. All the lipid variables in Table 1 were significantly different between the two groups.

The frequency of the *MTHFR* mutation was determined by PCR and restriction digestion with *Hin*fI because the presence of the mutant residue (valine) creates an *Hin*fI recognition sequence. Table 2 indicates the allele and genotype frequencies in patients and healthy subjects. The mutation was relatively common in both groups, with allele frequencies of ~36%. The frequency of the homozygous mutant genotype (V/V) in the patient group (14.5%) was higher but did not differ significantly from that in the control group (10.7%). Enzyme activity was examined in 96 of the CAD patients

TABLE 2. Frequencies of *MTHFR* Normal (Alanine) and Mutant (Valine) Alleles and of Three Genotypes (A/A, V/A, and V/V) in Healthy Subjects (n=121) and CAD Patients (n=152)

MTHFR	Healthy Subjects	CAD Patients
Normal allele A	155/242 (64%)	192/304 (63.2%)
Mutant allele V	87/242 (35.9%)	112/304 (36.8%)
A/A	47/121 (38.8%)	62/152 (40.8%)
V/A	61/121 (50.4%)	68/152 (44.7%)
V/V	13/121 (10.7%)	22/152 (14.5%)



MTHFR thermolability and specific activity in CAD patients with the three *MTHFR* genotypes. ▲, +/+, V/V; ●, +/−, V/A; and □, −/−, A/A.

(79 men and 17 women). Mean MTHFR activity for the entire group was 30.1±12.1 nmol · h^{-1} · mg^{-1}, and mean residual activity after preheating at 46°C for 5 minutes was 34.1±11.7%. These values did not differ significantly between men and women (specific activity, 31.0±11.7% for men and 26.0±13.5% for women; residual activity, 34.8±11.3% for men and 31.1±13.8% for women).

To determine the effect of genotype on enzyme activity, the CAD group was divided into the three possible genotypes for the alanine-to-valine mutation (A/A, V/A, and V/V) (Figure). Homozygotes for the wild-type allele had a mean MTHFR activity of 33.6±7.5 nmol · h^{-1} · mg^{-1}, with a mean residual activity after heating of 42.1±6.0%. The homozygotes for the mutant allele had a mean specific activity of 13.0±3.1% with a mean residual activity of 11.4±6.9%. The heterozygotes had values that were intermediate between the above two groups: mean specific activity of 24.3±5.8% and mean residual activity of 33.7±6.6%. The differences between all groups (pairwise) were significantly different with respect to specific activity and residual activity ($P<.001$). In all but one homozygote for the mutation, <20% of the initial enzyme activity was observed after heating, and all patients with <20% residual activity were homozygous mutant. Specific MTHFR activity was positively correlated to the residual activity ($r=.81$, $P<.001$).

To examine the influence of the genotype on plasma homocysteine, the sample of women was excluded because men and women have been reported to have different values for plasma homocysteine,[12] and there were insufficient numbers of women with CAD in our study (n=31) for statistical analysis after dividing them into the three possible genotypes. Table 3 indicates the plasma homocysteine and folate levels by genotype for healthy men and men with CAD. The homozygous mutant genotype was associated with higher levels of plasma homocysteine than the normal genotype; the difference was statistically significant in the CAD group ($P<.05$). Folate levels were not significantly different by genotype in control subjects and men with CAD. There were no differences in the other vitamins (B_{12} and pyridoxal phosphate) or in the lipid variables between genotypes in the men with CAD (data not shown). There was no correlation between age and homocysteine or folate levels in our groups.

Because a mutation in *MTHFR* might alter folic acid requirements in mutant individuals, we divided the samples on the basis of plasma folate, using the median value as the cutoff for each group (3.8 ng/mL for the CAD group and 3.3 ng/mL for healthy men). Table 4 illustrates the homocysteine level by genotype in the groups divided by folate status. In individuals with folate levels above the median, there was no significant difference between genotypes. However, the difference between genotypes was maintained and the homocysteine levels became even more pronounced in the groups with folate levels below the median. These data suggest an interaction between the homozygous mutant genotype and folate status in the elevation of plasma homocysteine.

Discussion

Although mild hyperhomocysteinemia has become recognized as a risk factor for occlusive arterial disease, the contribution of genetic factors to this process has not been studied extensively. Inborn errors of metabolism that lead to hyperhomocysteinemia and homocystinuria have provided some insight into the genetic mechanisms that might be important. Heterozygosity for deficiency of CBS, the first enzyme in the transsulfuration pathway of homocysteine metabolism, had been postulated as a risk factor for mild hyperhomocysteinemia[2,4]; however, a recent report suggests that CBS deficiency is not common in the vascular disease population.[8] The alanine-to-valine mutation in *MTHFR*, a missense mutation that results in a thermolabile variant of the enzyme, is the only common genetic change identified thus far that is associated with mild hyperhomocysteinemia.

In the present study, we identified the mutation in 36% of alleles, substantiating our initial report on the frequency of this polymorphism in a French-Canadian population.[11] Fourteen percent of the CAD group were homozygous for the mutation whereas 10% were ho-

TABLE 3. Plasma Homocysteine and Folate Levels in Healthy Males and in Male CAD Patients as a Function of *MTHFR* Genotype

	Healthy Subjects		CAD Patients	
Genotype	Homocysteine, μmol/L	Folate, ng/mL	Homocysteine, μmol/L	Folate, ng/mL
A/A	10.2±5.2 (n=31)	4.3±2.9 (n=31)	10.3±2.2 (n=56)	5.2±3.5 (n=39)
V/A	10.5±4.2 (n=47)	3.7±2.0 (n=47)	11.5±3.5 (n=51)	4.3±2.0 (n=43)
V/V	12.9±5.3 (n=8)	3.4±2.1 (n=8)	12.7±4.3 (n=14)*	4.7±4.3 (n=12)

Each value represents the mean±SD.
*$P<.05$ when homozygous mutant genotype V/V is compared with normal A/A genotype.

TABLE 4. Plasma Homocysteine in Healthy Men and in Men With CAD as a Function of *MTHFR* Genotype and Folate Levels

	Healthy Subjects		CAD Patients	
Genotype	Folate <Median	Folate >Median	Folate <Median	Folate >Median
A/A	10.6±3.6 (n=13)	10.1±6.2 (n=18)	11.3±2.4 (n=16)	9.6±2.1 (n=23)
V/A	10.1±3.7 (n=25)	11.0±4.8 (n=22)	12.1±2.9 (n=25)	10.2±1.9 (n=18)
V/V	15.8±4.6 (n=5)	8.2±1.4 (n=3)	14.6±5.1 (n=6)*	11.0±3.7 (n=6)

Each value represents the mean±SD.
*$P \leq .05$ when homozygous mutant genotype V/V is compared with normal A/A genotype.

mozygous mutant in a group of healthy subjects. Heterozygotes accounted for 45% and 50% of individuals in CAD and control groups, respectively. These values are similar to those we reported for a group of American individuals,[19] suggesting that these percentages are not unique to French Canadians. The mutation in the heterozygous or homozygous state significantly reduced MTHFR activity as well as residual activity after heating, with heterozygotes having values that were intermediate between those of normal individuals and homozygous mutant individuals. Because MTHFR synthesizes the major carbon donor for homocysteine remethylation, deficient activity would be expected to affect homocysteine metabolism.

The homozygous mutant genotype is associated with higher levels of homocysteine, particularly when plasma folate levels are in the low-normal range. The interaction between the genotype and folate status was first reported in our study of American individuals with undetermined clinical status.[19] The confirmation of that observation for CAD patients in the present study suggests a rational therapy, folate supplementation, for maintaining normal homocysteine levels in the presence of the mutation in individuals with vascular disease. Several studies have suggested that folate supplementation lowers homocysteine levels,[20] including a report in individuals who had thermolabile reductase activity.[21] Our data offer a genetic rationale for these earlier observations.

It is possible that the mutation itself may affect plasma folate levels, because 5-methyltetrahydrofolate is the primary circulatory form of folate. In severe MTHFR deficiency, with pediatric or adolescent onset of symptoms, the proportion of intracellular folate that is 5-methyltetrahydrofolate has been shown to be reduced, at least in fibroblasts.[22] Although the differences in plasma folate in the present study are not statistically significant between genotypes, homozygous mutant individuals do have lower plasma folate than normal individuals (Table 3). Although our study included a group of individuals without clinical evidence of vascular disease, they were not matched to the CAD group. Unlike the CAD group, they were a younger group of individuals who were not examined angiographically. The finding of elevated homocysteine levels in this group of healthy individuals with the homozygous mutant genotype may relate to these comments. These individuals may be at risk for vascular disease by virtue of their *MTHFR* genotype and folate status. Other as-yet-unidentified factors may contribute to the clinical outcome in this group.

In a study of Dutch patients with various forms of vascular disease, Kluijtmans et al[23] reported a threefold increase in the prevalence of the homozygous mutant genotype in a small group of patients (n=60) compared with control subjects. Our study examined only patients with CAD. Furthermore, unlike the above study, we did not exclude patients with other risk factors (hyperlipoproteinemia and hypertension, for example) for vascular disease.

Our findings suggest that the homozygous mutant genotype, in combination with low folate status, may predispose to hyperhomocysteinemia. Another variable that could contribute to hyperhomocysteinemia and vascular disease in this group of patients is the pyridoxal phosphate level, because this vitamin B_6 metabolite was present in significantly lower amounts in the patients than in the healthy individuals (Table 1). As suggested in a recent study,[24] low pyridoxal phosphate may confer an independent risk for CAD. In individuals in whom the remethylation pathway is compromised by *MTHFR* mutations or low folate, the transsulfuration pathway may be particularly sensitive to levels of PLP, the cofactor for CBS. Regulation of homocysteine metabolism can occur through SAM, which is synthesized from methionine; SAM is an inhibitor of MTHFR and an activator of CBS. If homocysteine remethylation to methionine is disturbed, a reduction in SAM levels might lead to decreased activation of CBS, which, combined with low PLP, might increase homocysteine accumulation.[25] Consequently, therapeutic intervention for hyperhomocysteinemia may require a multivitamin approach.

The identification of a common mutation of the *MTHFR* gene that has an impact on plasma homocysteine levels in the presence of low plasma folate levels is an example of gene-environment interaction. This finding suggests that a genetic predisposition to elevated homocysteine levels may be compensated for with folate supplementation. It remains to be determined whether supplemental folate or vitamin B_6 (or both) reduces cardiovascular risk. Clinical intervention trials are urgently needed to address this critical issue.

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